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Enantiomeric Degradation of 2-(4-Sulfophenyl)Butyrate via 4-Sulfocatechol in *Delftia acidovorans* SPB1

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Enrichment cultures with enantiomeric 2-(4-sulfophenyl)butyrate (SPB) as the sole added source(s) of carbon and energy for growth yielded a pure culture of a degradative bacterium, which was identified as *Delftia acidovorans* SPB1. The organism utilized the enantiomers sequentially. *R*-SPB was utilized first (specific growth rate [μ] = 0.28 h^{-1}), with transient excretion of an unknown intermediate, which was identified as 4-sulfocatechol (4SC). Utilization of *S*-SPB was slower (μ = 0.016 h^{-1}) and was initiated only after the first enantiomer was exhausted. Suspensions of cells grown in *S*-SPB excreted 4SC, so metabolism of the two enantiomers converged at 4SC. The latter was degraded by *ortho* cleavage via 3-sulfo-*cis*,*cis*-muconate. Strain SPB1 grew with 4SC and with 1-(4-sulfophenyl)octane (referred to herein as model LAS) but not with commercial linear alkylbenzenesulfonate (LAS) surfactant, which is subterminally substituted but nontoxic. It would appear that metabolism of the model LAS does not represent metabolism of commercial LAS.

One of the major xenobiotic compounds degraded in sewage works worldwide is linear alkylbenzenesulfonate (LAS) surfactant, which has an annual production of about 2,500,000 tons (40). Whereas the degradation of LAS is well established (16, 26, 27, 35, 39, 43), the mechanisms of its degradation are not (4).

Commercial LAS (Fig. 1) is, ideally, a mixture of linear alkanes (C_{10} to C_{13}) subterminally substituted with a single 4-sulfophenyl moiety (31): this means a mixture of 20 compounds, 18 of which are racemic mixtures. Although many different degradative reactions have been deduced from the intermediates which have been observed (38, 43), the general opinion is that the major degradative pathway involves oxygenation of one or both of the terminal methyl groups by an unknown oxygenase (see also references 19 and 20) followed by rounds of β-oxidation to yield a large set of transient sulfophenyl(di)carboxylates (Fig. 1) (9, 14, 17, 38, 43, 47, 49). On paper, seven sulfophenyl(di)carboxylates, representing a maximum number of β-oxidation steps, can be proposed: 2-(4-sulfophenyl)butyrate (SPB) (which is often observed during the degradation of commercial LAS [e.g., see reference 14]), 2-(4-sulfophenyl)propionate, 2-(4-sulfophenyl)malonate, 3-(4-sulfophenyl)butyrate, 2-(4-sulfophenyl)succinate, 3-(4-sulfophenyl)valerate and 3-(4-sulfophenyl)glutarate.

Many of these sulfophenyl(di)carboxylates are optically active (24), which can be assumed to have consequences for their further degradation (25, 50). SPB is quantitatively converted to cell material, CO_2, water, and sulfate by activated sludge (27), but none of the degradative mechanisms are known (4).

We now describe the isolation of *Delftia acidovorans* SPB1, which degrades both enantiomers of SPB via 4-sulfocatechol (4SC) and *ortho* ring cleavage (7, 13).

MATERIALS AND METHODS

Materials. Racemic, *R*- and *S*-SPB were synthesized by reacting sulfuric acid with the corresponding phenylcarboxylic acid (45). Yields of about 30% were obtained at about 97% purity; the major by-product, 2-(2-sulfophenyl)butyrate, was not totally removed on recrystallization (retention time, about 4 min [Fig. 2]). The identity of the product was confirmed by electrospray mass spectrometry (MS) and nuclear magnetic resonance. The following mass spectral data were collected in the negative ion mode: $[M - 2H]^{2-}$, *m/z* 121; $[M - H]^-$, *m/z* 243; $[M + Na^+ - 2H]^-$, *m/z* 265. ^1H nuclear magnetic resonance measurements gave four sets of peaks with chemical shifts downfield of tetramethylsilane: 0.87 ppm (t, 3H), CH_3 (C-4); 1.85 ppm (m, 2H), CH_2 (C-3); 3.47 ppm (t, 1H), CH (C-2); 7.6 ppm (pq, 4H), aromatic protons indicating *para* substitution of the sulfonate group (34). Synthetic *R*-SPB was obtained at 96% purity and an enantiomer excess of 96%; *S*-SPB was 96% pure with an enantiomer excess of 94%.

4SC was kindly supplied at about 95% purity by B. Feigel (13); the major impurity was 3-sulfocatechol. 3-Sulfocatechol was available from F. Junker (22). The identity of 4SC was confirmed by electrospray MS in the negative ion mode, where $[M - H]^-$ *m/z* 189 was observed. Commercial LAS (Marlon A 350) was kindly supplied by Hüls, Marl, Germany. The starting materials for the chemical syntheses were purchased from Fluka (Buchs, Switzerland). Other chemicals were from Fluka, Aldrich (St. Louis, Mo.) or Merck (Darmstadt, Germany).

Intermediates in the degradation of LAS, presumably sulfophenylcarboxylates and sulfophenyldicarboxylates (based on their UV spectra), were generated in a laboratory trickling filter during its early stages of use, as indicated elsewhere (27). The eluate from the trickling filter was chromatographed (26), the fractions corresponding to the desired material were collected and subjected to solid-phase extraction to remove the perchlorate in the mobile phase (28), and the neutralized material was used in growth medium.

Three sources of inocula were used in the enrichment cultures: activated sludge from the sewage treatment plants in Radolfzell (largely communal wastes), Konstanz (communal and some industrial wastes), and Ludwigshafen (largely industrial wastes), all in Germany.

The reference culture for the degradation of 4SC via *ortho* ring cleavage is a mixture of *Hydrogenophaga palleronii* S1 and *Agrobacterium radiobacter* S2 (18), and each was kindly made available by A. Stolz, University of Stuttgart, Stuttgart, Germany.

Analytical methods. Racemic SPB was initially detected by reversed-phase high-performance liquid chromatography (HPLC) (8, 32). When it became clear that a chiral separation was required, we chose a β-pm-Nucleodex column (5-μm diameter particles; 200-mm column of 4-mm internal diameter; Macherey & Nagel, Düren, Germany). We found 100 mM potassium phosphate buffer, pH 6.0, to give optimal separation and operated the column at 0.5 ml/min at room temperature (about 23°C). The sample volume did not exceed 50 μl. The diode array detector was set to give a chromatogram at 220 nm. *R*-SPB eluted at 6.5 min with baseline separation from *S*-SPB (8.4 min) (Fig. 2). The *ortho* analogues, impurities from the synthesis, eluted at about 4.1 min, whereas 4SC eluted at 4.5 min (Fig. 2). The standard curve for *R*-SPB, *S*-SPB, or 4SC was linear to about 40, 40, or 10 nmol/injection, and the limits of detection were about 0.25, 0.25, and 0.05 nmol/injection, respectively. The reproducibility of quantitation (peak area) for the SPB enantiomers was 98.8% (n = 6), and that for 4SC was 97.2% (n = 6). The retention times at constant temperature varied by ±0.05 min.

Oxygen uptake was measured in a Clarke type oxygen electrode (Rank, Cam-

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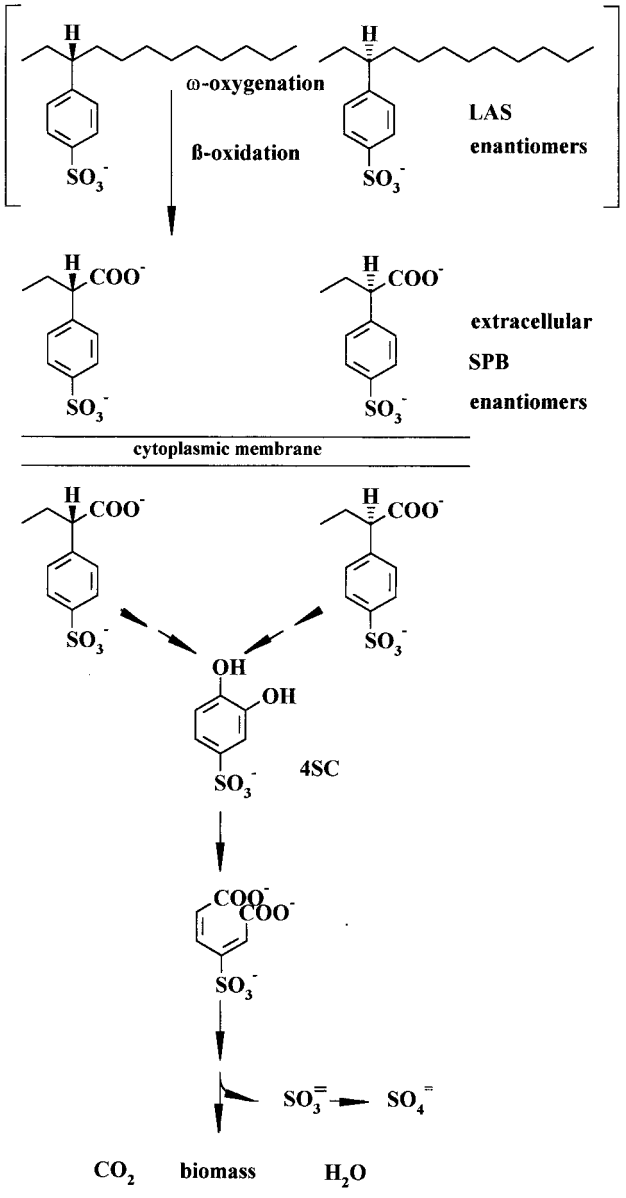

FIG. 1. Presumed major pathway for the conversion of racemic, commercial LAS to racemic sulfophenylcarboxylates, with an example yielding the two enantiomers of SPB, which are converted to 4SC and subject to *ortho* ring cleavage and desulfonation by *D. acidivorans* SPB1. The square brackets represent metabolism in another portion(s) of the putative community which degrades commercial LAS (21, 36a, 41). SPB represents one transient intermediate found extracellularly in sewage works, receiving waters, and soil (9, 14, 17). It is then sensible to anticipate the transport of SPB into the cell, because all sulfonates require transport systems (7). The mechanism of conversion of SPB to 4SC is still unknown. SPB is one of the seven smallest sulfophenyl(di)carboxylates which can be derived from LAS.

bridge, United Kingdom) with a 0.5-ml working volume. Sulfate ion was detected turbidimetrically after precipitation with barium ion (44). Sulfite was detected colorimetrically (29). Protein assay was performed by a Lowry method (6). Dissolved organic carbon was detected in a total organic carbon analyzer (15). Aromatic ring cleavage for taxonomic purposes was deduced by standard methods (42). A partial 16S rRNA gene sequence (450 bp) of strain SPB1 was determined by the German Culture Collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), where the sequence data were aligned and compared as described elsewhere (33, 36).

Enrichment cultures and growth of organisms. Aerobic enrichment cultures were set up with inocula from three activated sludges. Cultures (5 ml) in 30-ml screw-cap tubes were incubated at 30°C on a roller in the dark. The substrate, racemic SPB as the sole source of carbon and energy for growth, was present at 6 mM in a salts medium buffered with 50 mM phosphate, pH 7.2 (46). Cultures were streaked on Plate Count Agar (Difco) after at least three passages in the enrichment medium. Individual colonies were picked and removed to selective liquid medium. Positive cultures were restreaked and subcultured in selective liquid medium. A culture was considered pure when three successive plates were homogeneous and identical. Short-term storage of the organisms was on selective medium solidified with 1.5% agar as slants at 4°C. Long-term storage was in 50% glycerol at −80°C. The organism was deposited with the German Culture Collection under the accession no. DSM 12586.

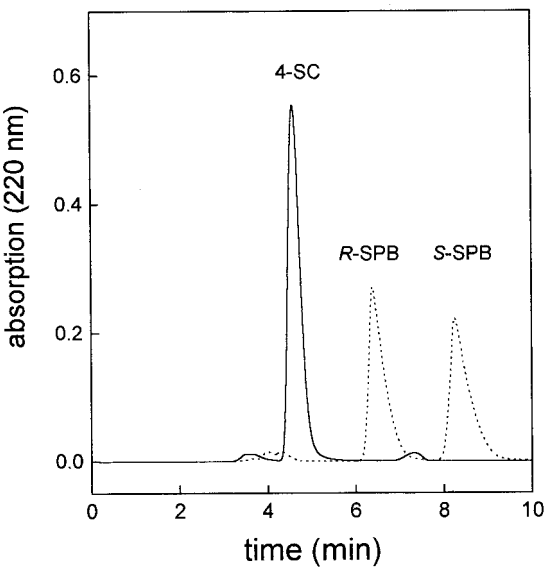

FIG. 2. Separation of *R*- and *S*-SPB from one another, from traces of the impurity, racemic 2-(2-sulfophenyl)butyrate, and from 4SC.

Growth experiments were done routinely in 400-ml cultures in 1-liter flat-bottomed, sidearm flasks mounted in a 30°C water bath. Aeration was by a magnetically driven stirring bar; sterile air was sparged through a washbottle at 30°C. Samples were taken at intervals through the side arm. Whole cultures, harvested before the end of growth and washed in 50 mM phosphate puffer, pH 7.2 (46), were used for the preparation of cell extracts. Growth of cultures utilizing enantiomers of SPB was carried out in 50-ml cultures stirred in 300-ml Erlenmeyer flasks with a sidearm in the form of a test tube; the test tubes fitted a photometer and allowed the optical density to be monitored without sacrificing material. Samples (1 ml), taken at intervals to measure the oxygen uptake of whole cells, were centrifuged (10,000 × *g* for 5 min at about 20°C) and the supernatant fluid was discarded; the cells were resuspended immediately in 0.5 ml of 50 mM potassium phosphate buffer, pH 7.5, and washed (10,000 × *g* for 5 min at about 20°C). The cells were resuspended in fresh potassium phosphate buffer and examined immediately for racemic SPB-, *S*-SPB-, or 4SC-dependent oxygen uptake.

Cultures of strain SPB1 were also grown with 10 mM succinate as the sole source of carbon and energy.

H. pallereronii S1 and *A. radiobacter* S2 were grown in 3 mM sulfanilate-salts medium (46).

Cell extracts. Cells in the mid-exponential phase of growth with *R*-SPB in the racemic mixture (or sulfanilate) were harvested, washed twice in 50 mM potassium phosphate buffer, pH 7.2, and resuspended for cell rupture in a French press as described elsewhere (23). Some experiments to monitor the disappearance of 4SC were done in 1-ml reaction mixtures in a 1-cm light path quartz cuvette in a Uvikon 922 spectrophotometer, and UV spectra from 200 to 400 nm were taken at intervals. The reaction mixture (13), which contained 0.3 mg of protein (crude extract, supernatant fluid), was buffered with 50 mM Tris-HCl, pH 8.0, and the reaction was started by the addition of 150 nmol of 4SC. In some experiments, samples were taken at intervals and examined by HPLC.

RESULTS

Enrichment cultures, isolation, characterization, and identification of *D. acidovorans* SPB1. Enrichment cultures for organisms which could utilize racemic SPB (three of five positive) were obtained easily. Growth (turbidity) of the first culture within 1 week was normal, and subcultures usually grew

overnight. Three mixed cultures, SPB1 (from Radolfzell), SPB2 (from Konstanz), and SPB3 (from Ludwigshafen), were essentially homogeneous (by light microscopy) and comprised largely a single bacterial morphotype with some protozoan grazers. Strain SPB1 was readily isolated from the mixed culture of the same name. Isolation of a bacterium from cultures SPB2 and SPB3 was not achieved.

Strain SPB1 was an aerobic, motile, oxidase-positive, catalase-positive, gram-negative rod, which usually occurred in pairs. There was *ortho* cleavage of protocatechuate. Analysis of the sequence of the 16S rRNA gene indicated 99% identity with *D. acidovorans*, which is in agreement with the simple taxonomic tests done.

Growth characteristics of *D. acidovorans* SPB1. Preliminary growth experiments in racemic SPB-salts medium were done with suboptimal aeration. The culture developed a transient pink or purple color for several hours, during which time the *R* enantiomer disappeared. The *S* enantiomer then largely disappeared without significant growth. The coloration was interpreted as the iron complex of a catechol (12), and chromatography on the chiral column yielded a peak at 4.2 min, which coeluted with authentic 4SC and had the same UV spectrum as the standard (pH 6; see the legend to Fig. 4). Cochromatography of the unknown with 4SC was also observed on a reversed-phase column, where the (different) UV spectrum at pH 2 was identical for the unknown and the standard. A portion of the unknown was separated by HPLC and collected, and its UV spectrum was analyzed at pH 13. We thus have five properties which together identify the unknown as 4SC.

With improved aeration, the growth of strain SPB1 was found to accelerate to reach a specific growth rate of 0.28 h^{-1} (Fig. 3a). After about 32 h, the growth rate decreased to about 0.016 h^{-1} for about one generation. The first phase of growth was essentially concomitant with the disappearance of *R*-SPB and with the formation of sulfate; the transient excretion of 4SC was very limited (Fig. 3b). The molar growth yield was 6 g of protein/mol of C. The second growth phase involved quantitative utilization of *S*-SPB and was concomitant with the release of sulfonate-sulfur as sulfate (Fig. 3b). The molar growth yield in this phase of growth was about 4 g of protein/mol of C, lower than the yield observed in the first phase but in the normal range (5). An unknown product was excreted in this phase (λ_{max}, 201 nm; λ_{min}, 222 nm; λ_{max}, 228 nm; λ_{min}, 251 nm; λ_{max}, 283 nm; retention time on the chiral column, 6.5 min), but as the residual dissolved organic carbon was negligible (about 2% of the original carbon, corrected for the carbon in solution after growth with 4-hydroxybenzoate), we did not examine it further.

In similar growth experiments on a smaller scale with racemic SPB-salts medium or *R*-SPB- or *S*-SPB–salts medium, growth was observed in each case. Growth with *R*-SPB was accompanied by transient excretion of 4SC, as shown in Fig. 2. The *R*-SPB-dependent specific activity of oxygen uptake in whole cells reached a maximum well before the end of growth, whereas the specific activity of 4SC-dependent oxygen uptake was stable during the growth phases. The *R*-SPB-containing reaction mixtures in the oxygen electrode displayed an accumulation of 4SC, which was detected by HPLC. Growth of strain SPB1 in *S*-SPB–salts medium was apparently faster than in the second phase of growth in racemic SPB–salts medium, but no 4SC was excreted. Reaction mixtures with *S*-SPB-grown cells in the oxygen electrode accumulated 4SC from *S*-SPB to concentrations about 10-fold higher than the impurity with *R*-SPB, so 4SC is also an intermediate in the degradation of *S*-SPB.

Sulfite, the normal leaving group formed upon the degradation of sulfonates (7), was not detected during growth with racemic SPB. We presume there to be a sulfite oxidase which converts sulfite (perhaps intracellularly) to sulfate.

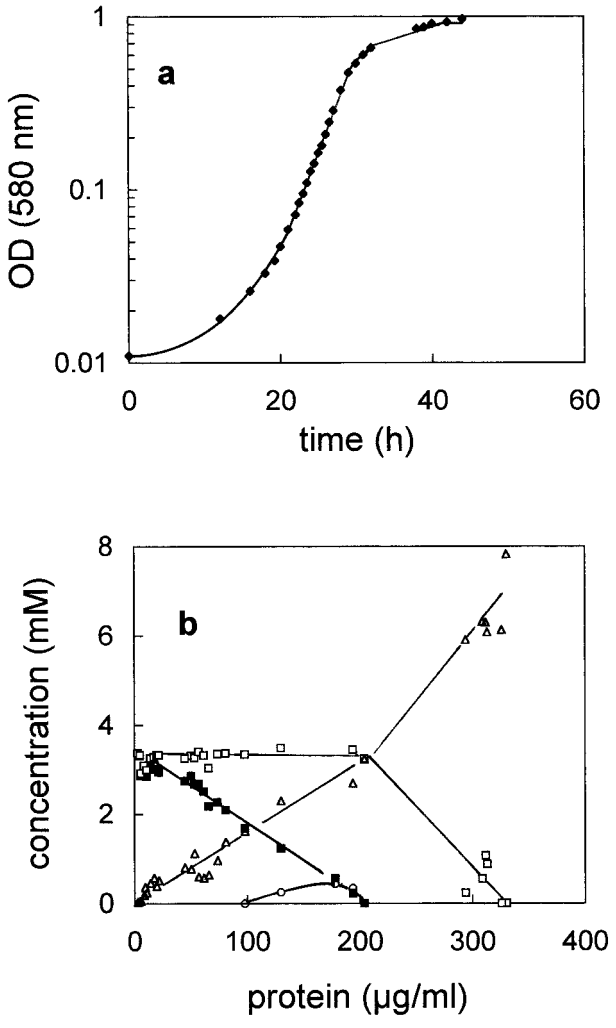

FIG. 3. Growth of *D. acidovorans* SPB1 with racemic SPB. Growth is shown in a conventional semilogarithmic plot (OD, optical density) (a) and in a linearized plot of the concentrations of substrates and products as a function of growth (b). Turbidity, ◆; *R*-SPB, ■; *S*-SPB, □; 4SC, ○; sulfate, △.

We examined the behavior of the mixed cultures SPB2 and SPB3 in SPB-salts medium. Utilization of both enantiomers was found in each case.

Ring cleavage of 4SC in crude extracts. Extracts of cells growing with *R*-SPB degraded 4SC (Fig. 4). No yellow color, which might indicate a *meta* cleavage, was detected, so we explored the possible presence of the *ortho* cleavage pathway, which was established by Feigel and Knackmuss (13). The pattern of biotransformation of 4SC (Fig. 4) is identical with that previously observed by Feigel (11). Samples taken during similar reactions and examined by reversed-phase HPLC showed the disappearance of the 4SC peak (at 2.2 min) and the appearance of a novel peak (at 1.9 min). In acidified samples, the novel peak disappeared and was replaced by another peak (at 2.1 min). This resembles the description of Contzen et al. (2), who found the elution of 3-sulfo-*cis*,*cis*-muconate prior to that of 4SC during reversed-phase HPLC and, after acidification, the spontaneous formation of the corresponding lactone,

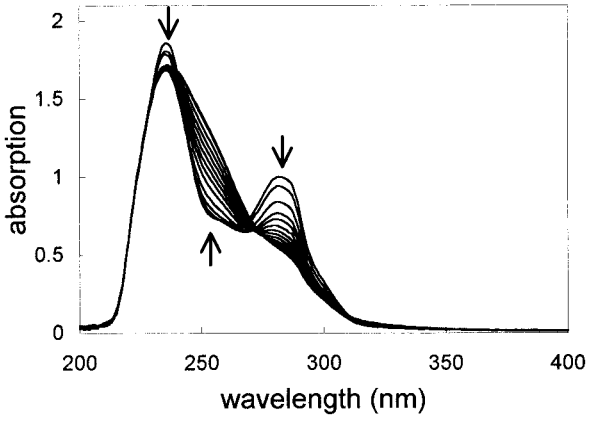

FIG. 4. UV spectra of the transformation of 4SC by crude extract of *D. acidovorans* SPB1. Spectra were taken at intervals; arrows pointing up or down indicate whether the absorption was increasing or decreasing. These conditions mask some of the spectral properties of 4SC. In our hands, 4SC at pH 2 displayed a maximum at low wavelengths (λ_{min}, 217 nm; λ_{max}, 233 nm; λ_{min}, 255 nm; λ_{max}, 279 nm). At pH 6 the maximum at low wavelengths was accompanied by values of λ_{min}, 226; λ_{max}, 234; λ_{min}, 267; and λ_{max}, 282 nm. At pH 13 the maximum at low wavelengths was accompanied by values of λ_{min}, 243 nm; λ_{max}, 266 nm; λ_{min}, 285 nm; and λ_{max}, 303 nm, similar to published data (48).

which had a longer retention time. The short retention times and interference from material in the enzyme assay made the measurement of UV spectra unsatisfactory, so we repeated the analyses on the chiral column. Our putative 3-sulfo-*cis*,*cis*-muconate coeluted (at 3.4 min) with authentic material from extracts of *H. palleronii* S1 and *A. radiobacter* S2, and both samples had the same UV spectrum in the diode array detector (a single maximum at 206 nm). Our putative lactone coeluted (at 4.1 min) with authentic material generated from the authentic sulfomuconate, and both samples had the same UV spectrum (with a maximum at 215 nm).

Substrate range of *D. acidovorans* SPB1. Strain SPB1 was able to grow with 4-hydroxybenzoate (6 mM) and 3,4-dihydroxybenzoate (3 mM) but not with 4-methylbenzoate (3 mM). The organism was found to utilize SPB, 4SC (3 mM), and 4-(1-octyl)benzenesulfonate (1 mM). There was no growth with 4-hydroxybenzenesulfonate (3 mM), 4-carboxybenzenesulfonate (3 mM), 4-methylbenzenesulfonate (3 mM), or commercial LAS (0.5 mM). With commercial LAS, the further addition of 4-hydroxybenzoate allowed growth but did not alter the foaming of the LAS, which obviously was not degraded.

A complex mixture of unidentified sulfophenylcarboxylate intermediates from the degradation of commercial LAS was collected. These compounds were not significantly degraded by strain SPB1, which did grow in their presence if a suitable carbon source was supplied. Sulfophenylcarboxylates were thus not toxic to this strain; they were simply not growth substrates. The mixed cultures SPB2 and SPB3 were capable of slight growth with the mixture of sulfophenylcarboxylates, but the disappearance of only one peak was detected in each case, so these organisms also have a narrow substrate range for sulfophenylcarboxylates.

DISCUSSION

The worldwide degradation of SPB, an intermediate from the degradation of LAS, has been implicitly recognized for many years (e.g., see references 17 and 43) and demonstrated directly in material from sewage works (e.g., see reference 27), so the isolation of a bacterium, *D. acidovorans* SPB1, able to degrade the compound is not surprising. What has been less widely considered is the importance of the chirality of molecules like SPB (Fig. 1 and 3), as emphasized by Kohler and coworkers (24, 50). Given that the subterminal substitution of alkanes is normal not only in LAS surfactants but also in the widespread secondary alkanesulfonate surfactants (30), the supposedly facile degradation of common household products is more complex than previously realized.

Growth of *D. acidovorans* SPB1, certainly with the *R* enantiomer, gives a normal value for the molar growth yield, which means a mass balance for carbon (see reference 3) as well as a mass balance for the sulfonate moiety (Fig. 3b). Utilization of the second enantiomer was initially not reproducible, and we attribute this to poor aeration, because improved aeration of flask cultures allowed full utilization of the second enantiomer with mass balance for the sulfonate moiety as sulfate (Fig. 3b). The growth yield with the second enantiomer was lower than that with the first, but as there was negligible residual carbon in the medium at the end of growth we presume the difference in yields to be facile, for example due to the increased contribution of maintenance energy to the energy budget at the lower growth rate (1). We also presume that an oxygenase attacking, e.g., *S*-SPB has a poor K_m for oxygen.

The growth yield, especially with *R*-SPB, indicates that all the carbon atoms in the compound are utilized for growth. Thus the side chain, as well as the ring, must be processed through the amphibolic pathways to generate carbon skeletons for biosynthesis and conservation of energy. We have, as yet, no information on the pathway involved in utilization of the side chain.

We presume the degradation of *R*- and *S*-SPB to be initiated by transport of these charged compounds across the cell membrane (see reference 7), and we hypothesize one transport system per enantiomer (see reference 50). The metabolism of the enantiomers converges at 4SC, because this compound is observed as a transient intermediate during growth with *R*-SPB (Fig. 3b) and in suspensions of cells from cultures utilizing *S*-SPB. We do not yet know how these compounds are converted to 4SC, whether in one novel dioxygenation or in several steps. We do, however, know that 4SC is degraded via the *ortho* cleavage pathway established for sulfanilate and benzene 1,3-disulfonate (2, 13) (Fig. 4). This new example of 4SC functioning as a point of convergence of metabolic pathways and as the substrate for ring cleavage points out the general importance of the previous work (2, 13), especially if the degradation of LAS is always channeled through this pathway.

This is the first pathway for the degradation of sulfophenylcarboxylate(s) from commercial LAS to be proposed with direct evidence from work with a pure culture. Many hypothetical pathways can be found in the literature (e.g., see references 37, 38, 43, 47, and 49), but none involves 4SC. When more isolates are available, we will be able to see whether any of the old hypotheses are correct.

One major dichotomy found in the literature on LAS is whether pure cultures can degrade LAS (49) or whether communities are needed (21, 41; see also reference 19). We suspect the difference lies in the LAS used. Commercial LAS is complex (see the introduction and Fig. 1) and is used by those who have found communities to be needed for complete degradation (10, 21, 41). Model LAS is commercially available as single compounds [e.g., 1-(4-sulfophenyl)octane], each a terminally substituted alkane, whereas commercial LAS is always subterminally substituted (Fig. 1). The published work which illustrates pure cultures (49) seems to involves model LAS. The isolate used in this paper, *D. acidovorans* SPB1, utilizes model LAS but not commercial LAS. So, we presume that the me-

tabolism of model LAS need not represent metabolism of commercial LAS. We have now isolated a heterotrophic bacterium which utilizes commercial LAS and concomitantly excretes sulfophenylcarboxylates of intermediate length (36a). Hrsak and Begonja (19), with a methanotroph catalyzing the oxygenation of commercial LAS to a sulfophenylcarboxylate of the same chain length, required a tier of heterotrophic organisms to generate compounds analogous to SPB. We presume that communities are generally required to degrade commercial LAS completely, as indicated elsewhere (21, 41).

The organism we isolated to degrade SPB, *D. acidovorans* SPB1, represents organisms widespread in soil and water, so the degradative pathway could be widespread in this species, or perhaps it is readily spread by horizontal gene transfer. Strain SPB1 has, however, a narrow substrate range for sulfophenylcarboxylates. Many intermediate mono- and dicarboxylated sulfophenyl moieties can be expected from LAS (9, 14, 17), yielding theoretically seven sulfophenylcarboxylates of minimal length, so if only organisms with a narrow substrate range degrade these compounds, the complexity of communities degrading LAS must be considerable. There are three pathways known to degrade 4-toluenesulfonate (7), so given that a higher tonnage of LAS is degraded annually (see the introduction), it is perfectly possible that other ring cleavage pathways are also involved.

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